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Determination of Alkaline Phosphatase Isozymes in Amniotic Fluid

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Summary: A simple method for the determination of the three isozymes of alkaline phosphatase (EC 3.1.3.1) contained in amniotic fluid (fetal intestinal, placental, and liver-bone-kidney) is presented. Total alkaline phosphatase activity was assayed in 10 000 *g* supernatants of amniotic fluid from 30 normal women between the 16th and 20th week of pregnancy. Electrophoretic patterns and inhibition by *L*-phenylalanine and *L*-homoarginine studies showed that all the fetal intestinal isozyme was precipitated in the pellet after centrifugation at 100 000 *g* for 90 min. Thus, the difference between total alkaline phosphatase activity and activity in the 100 000 *g* supernatant corresponds to fetal intestinal alkaline phosphatase. Placental isozyme can be determined by assaying alkaline phosphatase in the 100 000 *g* supernatant after heating at 56 °C for 90 min. Liver-bone-kidney isozyme activity is obtained by subtracting placental alkaline phosphatase activity from that of the 100 000 *g* supernatant. Mean percentages of the total alkaline phosphatase for each of the isozymes in amniotic fluid were 81% for fetal intestinal alkaline phosphatase, 7.5% for placental alkaline phosphatase and 12.0% for liver-bone-kidney alkaline phosphatase.
Determination of fetal intestinal alkaline phosphatase by this method could be applied to the diagnosis of cystic fibrosis in fetuses having a 1 : 4 risk of being affected.

Introduction

Human alkaline phosphatase (EC 3.1.3.1)[1]) exists as three isozymes with different tissue distribution: intestinal, placental and liver-bone-kidney. The three molecular forms can be identified on the basis of electrophoretic mobility, sensitivity to inhibitors, immunochemistry and heat stability (1). During the second trimester of pregnancy, amniotic fluid contains the three isozymes, although the intestinal form shows some differences to that found in adult intestinal microvilli. The fraction isolated from amniotic fluid is designated fetal intestinal alkaline phosphatase. The enzyme is located in the fetal small intestine mucosa. Since the microvilli are continuously desquamating into the lumen, their membranes and debris are incorporated into the meconium and normally pass to the amniotic fluid, especially during the second trimester of gestation (1). Fetuses affected by cystic fibrosis have a thickened meconium which slows down intestinal transit. Thus, less meconium is liberated to the amniotic fluid and the amount of fetal intestinal alkaline phosphatase in this fluid is substantially reduced (2, 3). Determination of activity in amniotic fluid between the 15th and 20th week of pregnancy has been proposed as a sensitive method for prenatal diagnosis of cystic fibrosis (4, 5). Thanks to the advances of molecular biology, other valuable diagnostic methods are available. Studies of restriction fragment length polymorphism of DNA segments of chromosome 7 indicated the existence of some "marker" restriction fragments linked to cystic fibrosis. Later isolation of the cystic fibrosis gene allowed a panel of probes to be obtained recognizing the most common mutations causing the disease. These techniques allow accurate diagnosis in a high percentage of cases (6–8). However, even admitting the possibility of false positive and negative results (about 3% and 8%, respectively) (4), enzyme determination in

[1]) Enzyme
Alkaline phosphatase (EC 3.1.3.1)

amniotic fluid is still a reliable method. It is approximately 90% accurate in cases of couples with cystic fibrosis and a 1 : 4 risk of having another cystic fibrosis baby (5). Presently available methods for fetal intestinal alkaline phosphatase determination involve use of monoclonal antibodies, availability of purified control isozymes or utilization of combined inhibitors, which complicate the procedure and are not simple enough for general use in the clinical laboratory (9, 10). We present here a method for the determination of isozymes of alkaline phosphatase in amniotic fluid and a simple technique for separation of fetal intestinal alkaline phosphatase.

Materials and Methods

Amniotic fluid

Thirty samples of amniotic fluid were obtained by amniocentesis of normal women being monitored for chromosomal abnormalities between the 16th and 20th week of pregnancy. The fluid was centrifuged at 1000 *g* for 10 min to separate suspended cells. The supernatant was centrifuged at 10 000 *g* for 15 min to precipitate remaining debris. A 4 ml aliquot of the supernatant was then centrifuged at 100 000 *g* for 90 min. The pellet was suspended in 0.2 ml of 50 mmol/l Tris-HCl buffer, pH 7.0 and used for enzyme assays and electrophoretic studies. A 2.0 ml aliquot of the supernatant was incubated in a water-bath at 56 °C for 90 min and then cooled on ice.

Intestinal tissue

Duodenum and jejunum were obtained within 24 h post-mortem at autopsy of adult human free of intestinal pathology. Mucosa was scrapped with a razor blade and stored at −23 °C.

Meconium

It was obtained immediately after birth of normal babies and stored at −23 °C until used for enzyme extraction.

Extraction of alkaline phosphatase

Fetal and adult intestinal alkaline phosphatase were extracted from meconium and adult mucosa, respectively by butanolic treatment following the technique described by *Morton* (11).

Enzyme assay

Alkaline phosphatase (EC 3.1.3.1) assay was performed at 37 °C in 2 ml of a reagent mixture containing 3 mmol/l *p*-nitrophenylphosphate, 0.5 mmol/l $MgCl_2$, 0.5 mmol/l diethanolamine pH 9.8 and 0.3 ml of the 10 000 *g* supernatant of amniotic fluid. Reaction was stopped after 30 min by addition of 3 drops of 400 g/l NaOH. Absorbance was read at 405 nm. All determinations were carried out by duplicate and the activity expressed in Units per l of sample. One Unit is the amount of enzyme catalyzing the conversion of 1 µmol of substrate per min in the assay conditions (12).

Electrophoresis

It was performed in 6.5% polyacrylamide gel with the micromethod described by *Ogita & Markert* (13) after addition of 1 g/l Triton X-100 to the preparation. The samples were run with 12.5 mmol/l Tris-glycine pH 8.3 during 90 min at 200 V and 20 mA per gel. After electrophoresis was performed, alkaline phosphatase isozymes were revealed by staining with a mixture containing: 58 ml 100 mmol/l Tris/HCl pH 9.0, 2 ml $MgCl_2$ 50 mmol/l, 50 mg α-naphthyl acid phosphate monosodium salt and 25 mg Fast Blue RR salt. The mixture was filtered and used immediately, staining was performed in the dark (14).

Results

Characterization of the alkaline phosphatase isozymes in amniotic fluid

The 100 000 *g* pellet suspended in 50 mmol/l Tris-HCl buffer pH 7.0 was submitted to electrophoresis after addition of Triton X-100. Specific staining for alkaline phosphatase revealed a zone of intense activity (fig. 1A). Without Triton X-100, all the alkaline phosphatase activity in the pellet is retained at the origin (fig. 1F). Pellet extracts with butanol at pH 5.0, run under same condi-

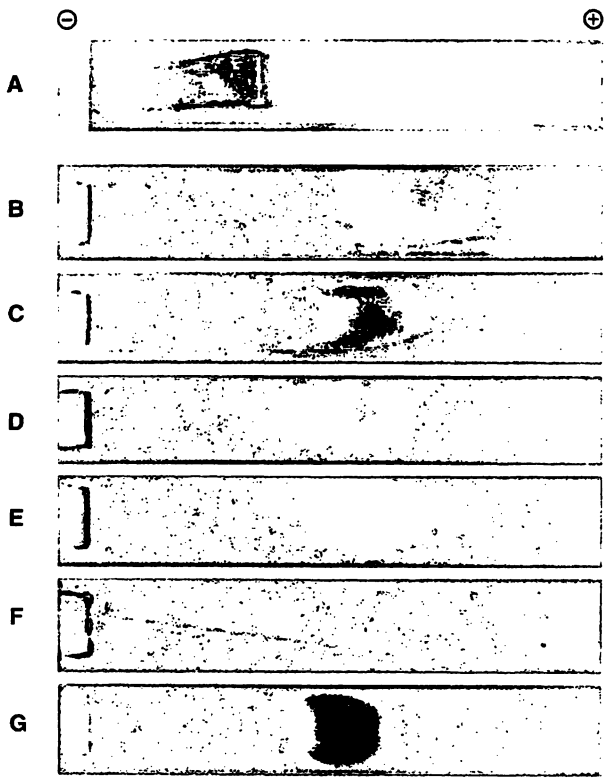

Fig. 1 Alkaline phosphatase electrophoretic patterns in polyacrylamide gels.
A – Suspension of amniotic fluid 100 000 *g* pellet treated with Triton X-100.
B – Butanolic extract at pH 5 from suspension of amniotic fluid 100 000 *g* pellet.
C – Butanolic extract at pH 5 from suspension of amniotic fluid 100 000 *g* pellet treated with neuraminidase.
D – Butanolic extract at pH 5 from meconium treated with neuraminidase.
E – Butanolic extract at pH 5 from meconium.
F – Suspension of 100 000 *g* pellet from amniotic fluid without Triton X-100.
G – Butanolic extract at pH 5 from adult intestinal tissue.

tions, showed a region of staining with a mobility about twice as fast as that of the original preparation treated with Triton X-100 (fig. 1B). When the pellet butanolic extract was previously incubated with neuroaminidase 0.2 kU/l at 37 °C for 2 h the mobility of the band was slightly reduced (fig. 1C). Alkaline phosphatase extracted from meconium by butanol at pH 5.0 treated and non-treated with neuraminidase presented the same velocity of migration as the band observed in the pellet preparations processed under the same conditions (fig. 1D and 1E). The enzyme extracted from adult intestinal mucosa gave a band with the same mobility as those in the amniotic fluid pellet and meconium after incubation with neuraminidase (fig. 1G). Electrophoretic studies of the amniotic fluid 100 000 *g* supernatant concentrated 20 times by ultrafiltration and incubated with neuraminidase to stress differences in mobility between the fetal intestinal alkaline phosphatase and the other isozymes present in amniotic fluid (placental and liver-bone-kidney alkaline phosphatase) revealed that no fetal intestinal alkaline phosphatase was present in the supernatant. Absolutely no activity could be detected in the region corresponding to fetal intestinal alkaline phosphatase (results not shown).

Assays of inhibition of the enzyme present in the pellet showed a behaviour similar to that of fetal (and adult) intestinal isozyme. In the presence of 2.5 mmol/l *L*-phenylalanine, the enzyme showed a 60% inhibition; that extracted from meconium 65% and that from adult intestine 62%. With 10 mmol/l *L*-homoarginine, inhibition was 25% for the enzyme in the pellet, 20% for that from meconium and 21% for that in adult intestinal mucosa. Inhibition of the alkaline phosphatase contained in the supernatant after centrifugation at 100 000 *g* was 52% with *L*-phenylalanine and 48% with *L*-homoarginine. Reported values of inhibition of the placental isozyme are 70% with 2.6 mmol/l *L*-phenylalanine and 9% with 10 mmol/l *L*-homoarginine and for liver-bone-kidney-isozyme, 9.5% and 80% for *L*-phenylalanine and *L*-homoarginine respectively (15).

Determination of alkaline phosphatase isozyme activity in amniotic fluid

The total alkaline phosphatase activity in amniotic fluid free of cells and debris (supernatant after 10 000 *g* centrifugation) gave a mean value of 25.0 (s. d. 6.6) U/l in 30 samples. This value corresponds to 100% activity. Since practically all the fetal intestinal alkaline phosphatase is separated in the 100 000 *g* pellet, the alkaline phosphatase activity of the supernatant after 100 000 *g* centrifugation subtracted from total activity gives the amount of intestinal alkaline phosphatase. The mean value for this fraction was 20.3 (s. d. 6.6) U/l, which corresponds to 81% of the total activity. It is known that the placental isozyme is more stable to heat than the other isozymes (16). Activity in the supernatant after heating at 56 °C for 90 min corresponds only to the placental alkaline phosphatase because the other isozymes are inactivated by such treatment. Its mean value was 1.85 (s. d. 1.35) U/l or 7.5% of the total. Liver-bone-kidney alkaline phosphatase activity is the difference between enzymic activity in supernatant minus remaining activity after heating (placental isozyme). Thus, the difference of alkaline phosphatase in the supernatant and placental isozyme corresponds to the liver-bone-kidney isozyme. The average value was 2.9 (s. d. 1.7) U/l or 12% of the total.

Discussion

The method presented allows determination of the three isozymes of alkaline phosphatase (fetal intestinal, placental and liver-bone-kidney) present in amniotic fluid. The proportion of each of the molecular forms obtained with this technique in amniotic fluid during the second trimester of pregnancy is similar to that found by other authors using immunochemical methods or combined inhibitors (9, 10).

The fetal intestinal alkaline phosphatase is the most abundant isozyme in amniotic fluid from the 15th to the 20th week of gestation. This form is associated with the microvilli which are continuously being sloughed and incorporated into the meconium and finally suspended in the amniotic fluid. This isozyme in the amniotic fluid is bound to membranous structures forming an aggregate or complex with a molecular mass greater than one million (14). As demonstrated by our results, this complex can be precipitated by centrifugation at 100 000 *g* for 90 min. Previously, we had communicated the separation of high M_r (biliary alkaline phosphatase), another membrane-associated alkaline phosphatase from serum of cholestatic patients, using a similar procedure (17).

The fraction obtained in the 100 000 *g* pellet of amniotic fluid showed the electrophoretic characteristics and inhibition by *L*-phenylalanine and *L*-homoarginine typical of fetal intestinal alkaline phosphatase. Absolutely no fetal intestinal alkaline phosphatase activity could be detected in the supernatant of 100 000 *g*. Conversely, only the fetal intestinal isozyme was found in the pellet, which did not show contamination with other isozymes.

Although the advances of molecular biology offer techniques with a high degree of accuracy, alkaline phosphatase determination in amniotic fluid is still a convenient method for most clinical laboratories.

The procedure presented here further simplifies the technique, since it does not require the use of control isozymes, monoclonal antibodies or use of combined inhibitors to determine the fetal intestinal alkaline phosphatase. Further investigation is required to assess the possible usefulness of this method for the prenatal diagnosis of cystic fibrosis.

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